

63.           **THE DETERMINATION OF CARCINOGENIC  
ACTIVITY OF CHEMICAL SUBSTANCES**

Many of the toxic effects of substances which might be used in food could be easily detected in man. For example, if a substance causes diarrhoea or is an inhibitor of choline esterase, its effects will be seen within one day and the relation between cause and effect can be recognised. The production of cancer or other long term effects is, however, difficult if not often impossible to detect in man, because the latent period for cancer induction in man appears to be at least twenty years in those cases in which we have evidence. Thus with bladder cancer occurring in the chemical industry and induced with 2-naphthylamine, the average time between first exposure to the chemical and the appearance of cancer is about eighteen years <sup>(1)</sup>. 2-Naphthylamine is a very active carcinogenic compound and other carcinogens probably take longer to act. The average latent period for lung cancer caused by cigarette smoking appears to be about thirty years.

The incidence of cancer caused by a chemical substance in the environment might be too low to detect and yet be important to the population. If the use of a product caused cancer in one person in each thousand of the population, this would be impossible to detect by any means available at present, but might be of great importance as a hazard.

The greater part of cancer in man could be due to external factors. In Britain some 40% of all cancer in the male is lung cancer and appears to be caused by cigarette smoking and there are indications that cancer of many different sites including the bladder, stomach, penis, cervix, skin and mouth are caused by environmental factors. The carcinogenic factors must be discovered and avoided. They can be discovered by epidemiological investigations and by laboratory research. To my mind there should be much more investigation into the causes of cancer, particularly by investigating the causes in man. Our knowledge of carcinogenesis depends fundamentally upon the cancers in man which have

developed following adventitious exposure to carcinogenic stimuli. These exposures have generally occurred in industry.

Most of the known compounds which cause cancer are synthetic products, products of combustion or materials associated with industry. Some natural products, however, are carcinogenic. Some of the Senecio alkaloids which occur in plants used in primitive medicine in Africa cause cancer of the liver, and the lactone, methyl proto-anaemin, found in some species of *Ranunculus*, causes cancer on injection (<sup>2</sup>). During the past year, it has been found the mould *Aspergillus flavus*, which infects and grows on ground nuts or pea nuts produces a substance, aflatoxin, which induces liver cancer when fed at low dose levels to rats (<sup>3</sup>). This finding is important and complicates the problem of food additives as this carcinogen which is produced by fermentation appears to be as potent as any synthetic substances which have been proposed as food additives, including 2-acetylaminofluorene.

Another interesting recent discovery is that of the high incidence of liver cancer in trout which are bred in America and are fed on artificial food. The high incidence of cancer in these fish appears to be due to some material, as yet unidentified, in the food and shows that food materials have caused cancer in animals.

Although we know much about chemical carcinogenesis, our knowledge is insufficient to predict with certainty whether a substance of known structure will be carcinogenic. Even in certain groups of compounds such as the polycyclic hydrocarbons, the azodyes and lactones we know sufficient to predict the activity, but not with sufficient certainty to make biological testing of a proposed food additive unnecessary.

There are as yet no completely acceptable rapid tests for carcinogenic activity, although some have been proposed and are under investigation. The parallelism between mutagenic activity and carcinogenesis is not sufficiently close to say that all mutagens are carcinogenic. Although the induction of cancer is probably a somatic mutation, the induction of mutations in invertebrates (eg: *Drosophila* and *Neurospora*), in which mutation can be measured, and in vertebrates probably have different requirements.

One quick test which has been proposed depends upon the effects on the limb buds of the newt, Triton, used by NEUKOMM (<sup>4</sup>) in Lausanne. This method takes a few days but it is difficult to know how completely the effect parallels that of carcinogenic activity.

In another relatively rapid method introduced by Rous and SMITH (<sup>5</sup>) the substance under investigation is implanted with some embryo tissue

into the muscle of mice or rats. The embryonic tissue responds quickly with known carcinogenic hydrocarbons, giving cancer of the embryo tissue in three months. This method needs investigation with other types of carcinogenic agents.

Although these quick tests for carcinogenic activity are of interest none of them can as yet replace the more conventional biological tests.

Many of the substances proposed as food additives are so called weak carcinogens. The activity may be so described in at least three different aspects. First the substance may be slow in action, thus 1:2-5:6-dibenzanthracene, which is carcinogenic in very small quantities, takes at least nine months to induce cancer in mice no matter how large a dose is administered; 9.10-dimethyl-1:2-benzanthracene on the other hand induces tumours in three months, but larger amounts are required. 1:2-5:6-Dibenzanthracene is therefore a slow acting carcinogen.

Secondly carcinogens may be described as weak because large doses are required. Thus to obtain tumours in all animals by treatment with the azo dye, Butter yellow, quantities of the order of a gram must be administered while 1:2-5:6-dibenzanthracene is active in microgram quantities. In this respect 1:2-5:6-dibenzanthracene might be considered to be a million times more active than the dye.

Thirdly some carcinogens are weak because they do not produce tumours in all animals even when given in big doses. Even large doses of 1:2-benzanthracene produce tumours in only about half of the animals (<sup>6</sup>). For assay of carcinogens of this type large numbers of animals may be necessary.

All these aspects of activity should be considered in carrying out biological tests, the time of cancer induction being very important. Whereas the latent period for cancer induction in man is about twenty years in mice it is generally less than one year. The tumour induction time varies with the usual life span of the species and it is for this reason that the short lived rodents are suitable for laboratory testing of carcinogenic activity. Rabbits and dogs which live for a decade or more need many years to develop cancer even with potent carcinogens.

All substances, with the exception of arsenic, which are known to cause cancer in man have induced cancer in at least one species when they have been adequately tested in animals. There is however considerable species variation. 2-Naphthylamine which causes cancer of the bladder in man and in dogs, induces liver tumours in mice. Butter yellow induced tumours more readily in rats than in mice. At least two species of animal should be used in tests with food additives, because

of the variation in response of different species. The British Ministry of Health (7) has made recommendations for the testing of carcinogenic substances and suggested that rats and mice are suitable. Other authorities have recommended that one of the species used should be a non-rodent. The only suitable non-rodent is the dog and the American authorities recommend the use of rats or mice and dogs. Dogs are not convenient because the expense and difficulty of maintaining them generally allows only small groups to be used, so that only a very high incidence of cancer could be detected with any certainty. They are also slow in responding to carcinogenic agents so that tests with dogs should be carried out for at least four or five years and possibly for seven years.

Experience with benzidine presents an example of the unsuitability of dogs. Benzidine is known to cause bladder cancer in man, but it has never been satisfactorily shown to be a carcinogen in dogs. Although bladder tumours appeared in two dogs after seven years of treatment adequate control animals do not appear to have been used in the experiment for one to say with certainty that benzidine causes cancer in dogs. On the other hand, benzidine induces tumours of the liver and other organs in rats (8).

This result shows that the same substance may induce bladder cancer in man and liver cancer in rodents and indicates that the induction of cancer of any particular organ in animals by a compound means that the compound may induce cancer either in the same organ or at some other site in man. Research is, however, still needed to find animals more suitable for carcinogenicity testing.

In addition to the species variations in carcinogenic response there are sex differences. Thus 2-acetylaminofluorene induces liver cancer more readily in male than in female rats. For this reason tests should be carried out on both sexes of animals.

The British Ministry of Health report recommended that substances be tested by feeding and by injection. There are however some substances which are not suitable for injection and in these cases the number of animals used in feeding tests should be greater. Examples of such materials are flour treated with improvers or milk which has been treated with hydrogen peroxide. There are sometimes difficulties in the interpretation of the significance of injection experiments. In the case of colouring matters the induction of cancer by injection is considered an indication that the material presents a hazard. This consideration depends upon the fact that the dyes being relatively small molecules, might be absorbed from the human alimentary tract, particularly as absorption can be

modified by other substances present in the diet. It is also based on the principle that if several substances are available with the same function, then the substance which is the least toxic should be used. The induction of cancer in rats by injection is an indication of toxicity.

In other cases such as the macromolecular cellulose derivatives including carboxymethylcellulose, the induction of tumours at the site of injection in rats is taken as an indication that the substance should be examined more thoroughly by feeding tests.

With food additives it appears unnecessary to carry out tests by painting or other methods of skin application although such tests might be of value in testing materials for use in cosmetics.

The sensitivity of the biological estimation for carcinogenic activity depends upon the numbers of animals used in each group. In order to obtain significant results, considerable numbers of animals are required to assay a substance which induces cancer in a small proportion of animals even when no tumours occur in the controls.

In the British report it was recommended that groups of twenty-five animals be used in each group at the beginning and that at least twelve mice of each sex should survive for eighty weeks and at least twelve rats of each sex survive for two years. These figures were based on injection and feeding experiments and the number of animals in each group should be increased if only feeding trials are performed.

There are differences of opinion as to the period during which the material under test should be administered. Some workers consider that the material should be fed for the entire duration of the test period. I think that it is preferable to feed the material for only half of the test period. One advantage of this is that reversible changes have time to disappear in the period during which the animals are kept after the dosing period. On the other hand, malignant changes will remain or develop. In the testing of aminotriazole which induced thyroid hyperplasia and thyroid tumours, the hyperplasia would have disappeared if the animals had been kept for a year after treatment. This would have made the diagnosis of the condition in the animals more certain.

I have previously argued (<sup>9</sup>) that from the point of view of evaluation of the hazard from possible food additives the only prudent assumption is that there is no safe dose of a carcinogen. Even if a safe or threshold level of a carcinogen exists, this would be difficult if not impossible to determine. With carcinogens in the human environment we are interested in low incidences of the disease — an incidence of one in one thousand must be considered dangerous. Not only is it impracticable

to determine the threshold in animal experiments, but it would be impossible, particularly at values for low incidence, to transfer this result to human experience.

There are theoretical reasons for the non-existence of a threshold dose of a carcinogen. In the case of many toxic agents which affect the liver many cells must be destroyed or injured before ill effects are produced. A liver poison may injure many million cells before an injurious effect on the body appears. On the other hand, the change of a single normal cell into a cancer can lead in the development of cancer and the death of the animal.

If one accepts this absence of a safe dose as a practical working guide, then in testing for carcinogenic activity it is possible to use only one dose level. There may be carcinogens for which an optimum dose level exists, but this is generally because the higher doses are toxic. In testing a carcinogen with one dose level, the level of carcinogen employed should be as high as possible, which will allow the animals to survive for sufficient time to develop tumours.

It is essential to have control groups of animals in tests for carcinogenic activity, as if susceptible animals are used, some tumours will probably develop in animals which are not treated with the suspected carcinogen. Groups of control animals must therefore be maintained under similar conditions as the experimental groups. The control groups must be at least as large as the experimental groups, particularly if the tests are to demonstrate that a substance is *not* carcinogenic.

The use of positive control animals is often advisable. These animals are treated with a known carcinogen which should if possible be of the same type as the material under test. The use of positive controls in this way ensures that the animals used could respond to a carcinogenic stimulus and also it permits the experiment to give some idea of the relative carcinogenic activity of the substance under test.

There are differences of opinion as to whether pure line animals should be used for testing. Such animals might be resistant to some particular carcinogenic agent but their response is likely to be more uniform than that of animals which are not genetically homogeneous. The use of  $F_1$  hybrids of pure line animals would probably offer advantages, but this is an aspect on which more investigation is needed.

The whole subject of the methods of testing for carcinogenic activity needs more research to develop and improve the existing methods. The attitude to this kind of work has changed during the past decade. At one time workers on carcinogenesis were more interested in demonstrat-

ing activity — now many of us are anxious to show that materials are safe. The proof of the negative — of absence of carcinogenic activity is more difficult and needs careful consideration and work.

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